Metabolite extraction from mammalian suspension cells using centrifugation for cell harvesting

Preparing cells for a metabolomics experiment

- Aim for an equal cell number per sample at the time of harvesting! You need at least 3 samples per condition.
- Plate about 3e5 – 1e6 cells per well in 6-well plates or 60 mm dishes or T25 flasks. For very small cell types like T-cells plate more, i.e. 2e6 -5e6 cells per sample.
- You can add an additional well/plate per condition for cell counting at the time of harvesting.
- Alternatively -or in addition-, you can determine the protein or DNA content of the sample pellets left over after extraction.

Preparing for a metabolomics experiment using isotope tracing

General considerations for stable isotope (e.g. C-13, N-15, H-2 (D)) labeling:

- For the labeling medium, obtain medium that does not contain the nutrient that is used as a tracer (i.e. glucose) and add the isotope tracer at the same concentration as in the regular medium (i.e., 5 mM U13C glucose instead regular glucose).
- The minimum labeling duration depends on the metabolic pathway of interest. For instance, metabolites in the glycolysis pathway only take 30 minutes to reach ***isotopic steady-state*** for many cell types, while other metabolic pathways (e.g. lipids) might take days. An 18-24 h labeling time is sufficient for steady state labeling of most central carbon metabolites.
- Ideally, use dialyzed FBS from which small molecules (metabolites) have been removed. Establish growth curves for the different cell lines using this dialyzed FBS, so that you can aim to have similar numbers of cells at the day of harvesting between conditions with different growth rates.

  Alternatively, you can use regular FBS, but maximum percent labeling will be lower since the FBS contains unlabeled tracer counterparts (i.e., glucose), effectively lowering the labeling percentage of the tracer.
- Plate the required number of cells in regular medium containing dialyzed FBS (if used) and incubate overnight.
- Next day, 3-24 h before extraction (depending on your desired labeling time), switch to medium containing the isotope tracer (and dialyzed FBS if used).

  A volume of 1.5-2 ml is sufficient to cover the cells in a 6-well plate for 24h, but keep in mind that the cells might deplete nutrients within that amount of time! In that case, replace a portion (i.e., 1/3th) of the medium 6 hours or so before harvesting.

Polar metabolites extraction from suspension cells using 80% MeOH

Notes:

- *Work on ice unless indicated otherwise.*
- *It is a good idea to take along one 'processing blank': process the same as the other samples, but without cells.*

1. Transfer the cells to a polypropylene centrifuge tube and spin for 5 min at 500-800 x g to gently pellet the cells. A swing-bucket centrifuge may be preferred to collect the cells on the bottom of the tubes.
2. Place the tubes on ice; aspirate the medium.
3. Wash the cells *briefly* by resuspending them gently in **ice-cold PBS or 150 mM ammonium acetate, pH7.4 solution** (*see notes below*).
4. Pellet the cells gently at 4 °C (using a swing bucket centrifuge).
5. Immediately place on ice and carefully aspirate the wash solution. Remove as much of the wash solution as possible without disturbing the pellet.

   ❄ *Cell pellet can be flash frozen in LN2 at this point for extraction at a later time. Store at -80C.*

6. Add **1 ml ice-cold 80% MeOH**/20% water to the cell pellets.

   Optional: use 80% MeOH containing 1 µM norvaline as a sample preparation internal control.

   ❄ *If cell pellets were stored at -80C, add the MeOH directly to the still frozen cell pellets.*

7. Vortex the samples for 30 sec.
8. Incubate the tubes for 30 min at -80 °C for effective extraction and protein precipitation.
9. Place on ice to warm up, then vortex the samples again.
10. Spin in a microcentrifuge at top speed (16,000 g) for 10 min at 4 °C.
11. Transfer the supernatants to glass vials (or new centrifuge tubes if a speed vac will be used).

    a) Keep the tubes with the pellets if normalization by protein content is desired. See step 14.

12. Dry the metabolite extracts down in an evaporator without applying heat – i.e., the Genevac EZ-2 Elite using program 3 (aqueous) or a speed vac. Choose an appropriate drying time for your sample volume and remove the samples promptly when the program is finished.
13. Store the dried extracts at -80 °C until ready for LC-MS analysis.

14. Determine protein content of the pellets:
    a) Briefly airdry the tubes to remove any remaining 80% MeOH from the tubes.
    b) Resuspend the pellets in relatively large volumes (i.e. 20 pellet volumes; i.e. 200 ul) of **0.2 M NaOH**
    c) Heat at 95 °C for 20 min. Flick the tubes to make sure the pellets are completely resuspended.
    d) Cool to RT. Spin the tubes for 15 min.
    e) Collect the supernatant and determine the protein content by **BCA method** using an i.e., 10-fold dilution.

    Of note, instead of protein content, the DNA content of the pellet can be determined using an appropriate resuspension solution.

Notes on various wash solutions for suspension cells:

For adherent cells, a **150 mM ammonium acetate** - a volatile salt - **solution** [(1)] is used for the washing step, because PBS - containing non-volatile salts - is incompatible with mass spectrometry.

However, suspension cells may be negatively affected by the NH4AcO wash. They can swell and deteriorate during the washing steps!

This is not the case when the cells are washed with **5% mannitol** [(2)]**.** See references below.
However, mannitol binds to the column, potentially affecting binding capacity and causing ion suppression of co-eluting metabolites (i.e., glucose in our LC-MS setup).

Thus**, PBS** may be the best washing solution, but …, the column flowthrough needs to be diverted to waste rather than going into the mass spec**, so make sure to mention that you used PBS to wash the cells!**

(1) 150 mM ammonium acetate solution, pH 7.4

Dissolve 1.1562 g ammonium acetate (Molecular Biology grade) per 100 ml of Millipore Milli-Q water (18 uΩ) to make a 150 mM solution.
Adjust the pH to 7.4 using a few drops of 1 M NH4OH (i.e., a 1:11 dilution of a 21% NH4OH stock solution).
If stored at room temperature, sterilize the solution using a Stericup Vacuum Filter Cup (i.e. Millipore Corp).

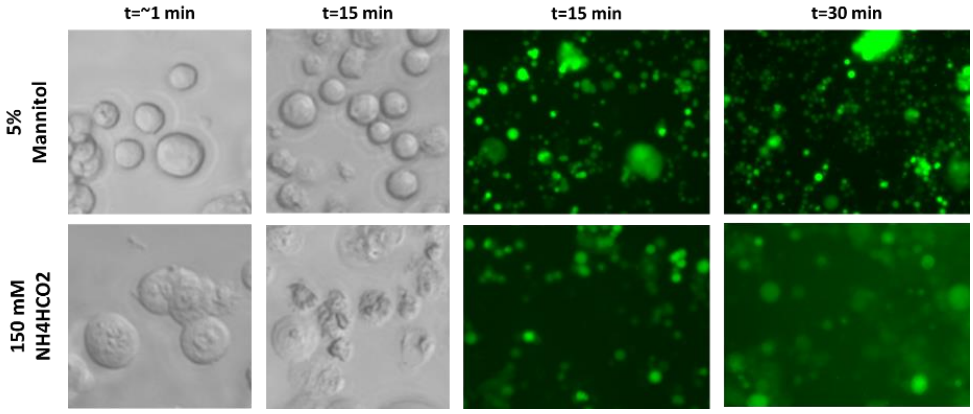
(2) 5% mannitol

Prepare 5% (w/v) mannitol by dissolving 5 g/100 ml Milli-Q water. Store short-term at 4 °C or autoclave.

References regarding the effects of a NH4OAc wash on suspension cells:

5% mannitol was used as an alternative.

- Palaskas et al. "Global alteration of T-lymphocyte metabolism by PD-L1 checkpoint involves a block of de novo nucleoside phosphate synthesis." *Cell Discov*\5, 62 (2019). doi:10.1038/s41421-019-0130-x
- Courtesy of Grigor Varuzhanyan, PhD at UCLA, using a GFP expressing prostate CA cell line:
  (The longer time points were used to show the effects more clearly)



Suggested (or similar) supplies needed for this protocol:

- Ammonium acetate	A1542-500G	FisherScientific	for molecular biology, ≥98%
- MeOH:	A456-1	FisherScientific	Fisher Methanol (Optima* LC/MS); 1L
- H2O:	W5-1	FisherScientific	Water, Glass Bottle; 1L
- Norvaline:	N7502-25G	Sigma	DL-Norvaline

Used as an internal standard: prepare a 100 mM solution in H2O. Make a 1 mM working stock in MeOH. Store both at -20C. Dilute 1:1000 for a 1 µM solution in 80% MeOH.

- glass vials:	13-622-351	FisherScientific	Chromacol™ GOLD-Grade Inert Glass Vials; Thermo 2SVWGK
- caps:	03-452-327	Fisher Scientific	9 mm Screw Caps, SureSTART™ Level 2 (Silicone/PTFE septum); Thermo Scientific 6ASC9STB1
	03-379-123	Fisher Scientific	9 mm autosampler vial screw thread caps (PTFE/Silicone septum); Thermo Scientific C500054A

Stable isotope-labeled tracers

Choose C-13- and/or N-15-labeled metabolites of high purity (≥ 98 % labeled)

- Cambridge Isotope Laboratories
  i.e., D-Glucose (U-13C6, 99%) CLM-1396-1 1 g
- Sigma
  i.e., D-Glucose-13C6 389374-1g 1 g